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AN ISOLATED YEAST PROMOTER SEQUENCE AND A METHOD OF REGULATED HETEROLOGOUS EXPRESSION

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This application is a continuation-in-part of application serial number 09/632,314 filed August 4, 2000.

FIELD OF THE INVENTION

The present invention relates specifically to the isolation of a yeast gene regulatory sequence (promoter), which is native to *Schwanniomyces castellii* (ATTCC 26077) and can regulate gene expression in a heterologous yeast host using starch as the sole carbon source. More specifically, the starch can be used as an inducing agent for the expression of native or foreign genes, which are fused to the isolated yeast promoter. The transformed host cells bearing the promoter-gene fusion can grow in culture medium containing various carbon sources, and the gene expression is induced by starch addition as a gene expression inducing-agent. The heterologous host is preferably bacteria, yeast, mold, plant cell, plant tissue and whole plant.

DESCRIPTION OF THE RELATED ART

Natural yeast strains have been identified that can use starch as a primary growth substrate via complete or partial enzymatic hydrolysis. These yeast strains include but not limit to *Saccharomycopsis fibuligera, Schwanniomyces castellii*, and *Saccharomyces diastaticus*, which can produce and secrete both alpha-amylase and glucoamylase to

liquefy and hydrolyze starch into glucose. A fusion yeast cell strain of *Saccharomyces diastaticus* and *Saccharomyces cerevisiae* could degrade 60% of starch present in culture media within two days. In addition, other natural *Saccharomyces* species can ferment starch and dextrin to ethanol, as well as improve ethanol production from starch and higher sugars.

The ability to genetically modify yeast strains has greatly advanced both protein expression engineering and metabolic engineering for the past two decades. The use of yeast for producing transgenic prokaryotic and eukaryotic heterologous proteins has the added advantage that yeast and filamentous mold are microbial eukaryotes and they are more closely related to animal cells. Hence, yeasts possess the molecular genetic manipulation and growth characteristics of prokaryotic organisms together with the subcellular machinery for performing eukaryotic post-translational protein modification. For example, *Pichia pastoris* is able to synthesize functional recombinant protein and its glycosylation abilities are very similar to those of animal cells, though the glycosylation in another yeast strain, *Saccharomyces cerevisiae* is different from that of an animal. In addition, the metabolic pathway of a regular ethanol producing yeast strain can be genetically altered to accumulate large amount of lactic acid, and to increase xylose utilization rate. However, only a few yeast systems (transformation vector and promoters) are available for protein engineering and metabolic engineering, which include *Saccharomyces cerevisiae*, *Pichia pastoris*, among others.

Starch utilizing yeast strain, *Schwanniomyces castellii* or *Schwanniomyces occidentalis*, is one of the most important microorganisms since it can directly use starch as its growth medium. Due to the low level of glycosylation and the ability of protein secretion, *Schwanniomyces castellii* is a promising host for heterologous protein expression. However, the molecular study of *Schwanniomyces sp.* is very limited. Only about 21 genes have been cloned and very few promoter sequences have been cloned and characterized in their full length from *Schwanniomyces sp.* The ability to genetically manipulate *Schwanniomyces sp.* depends on the successfulness in developing the transformation methods and gene expression systems. To effectively direct the transcription or expression of an interested gene, strong gene regulating elements or

promoters are required. These promoters can be isolated from the upstream sequences of strongly expressed gene clones.

Glucoamylase, a 146-kDa protein, is one of the highly expressed clones in *Schwanniomyces castellii*, and different carbohydrates such as maltose and starch regulate its expression. The expression level of glucoamylase can be increased by 100-fold when the cells are shifted from glucose culture medium to maltose culture medium. The gene regulatory element (promoter) of the glucoamylase gene would be a useful genetic element to be used for the regulation of foreign gene expression. However, the *Schwanniomyces castellii* glucoamylase promoter has never been fully sequenced and characterized. To genetically manipulate *Schwanniomyces sp*, either for the purpose of metabolic pathway modification, conferring necessary traits such as chemical production, or producing biocatalyst of interest, high levels of mRNA expression are always desirable. Therefore, there is a need to isolate strong promoter sequences of *Schwanniomyces sp.* and characterize its function.

The following references disclose technical information useful in this art:

Sills AM, Stewart GG. 1982. Production of amylolytic enzymes by several yeast species. *J. Inst. Brew.* 88: 313-316.

Hongpattarakere T, H-Kittikun A. 1995. Optimization of single-cell-protein production from cassava starch using *Schwanniomyces castellii. J. Microbiol. Biotechnol.* 11: 607-609.

Lemmel SA, Heimsch RC, Korus RA. 1980. Kinetics of growth and amylase production of Saccharomycopsis fibuligera on potato processing. *Appl. Environ. Microbiol.* 39: 387-393.

Kim K, Park CS, Mattoon JR. 1988. High-efficiency one-step starch utilization by transformed *Saccharomyces* cells which secrete both yeast glucoamylase and mouse alpha amylase. *Appl. Environ. Microbiol.* 54: 966-971.

Laluce C, Bertolini MC, Ernandes JR, Martini AV, Martini A. 1988. New amylolytic yeast strains for starch and dextrin fermentation. *Appl. Environ. Microbiol.* 54: 2447-2451.

Pirselova K, Smogrovicova D. Balaz S. 1993. Fermentation of starch to ethanol by a co-culture of *Saccharomycopsis fibuligera* and *Saccharomyces cerevisiae*. *World J. Microbiol. Biotechnol.* 9: 338-341.

Ryu YW, Ko SH, Byun SY, Kim C. 1994. Direct alcohol fermentation of starch by a derepressed mutant of *Schwanniomyces castellii*. *Biotechnol. Lett.* 16:107-112.

Sreekrishna K, Nelles L, Potenz R, Cruze J, Mazzaferro P, Fish W, Fuke M, Holden K, Phelps D, Wood P, Parker K. 1989. High-level expression, purification, and characterization of recombinant human tumor necrosis factor synthesized in the methylotrophic yeast *Pichia pastoris*. *Biochemistry* 28: 4117-4125.

Cregg JM, Vedvick TS, Raschke WC. 1993. Recent advances in the expression of foreign genes in Pichia pastoris. Biotechnol. 11: 905-910.

Porro D, Bianchi MM, Brambilla L, Menghini R, Bolzani D, Carrera V, Lievense J, Liu CL, Ranzi BM, Frontali L, Alberghina L. 1999. Replacement of a metabolic pathway for large-scale production of lactic acid from engineered yeasts. Appl. Environ. Microbiol. 65(9): 4211-4215.

Ho NWY. 1999. Successful development of hazard-free, user-friendly genetically engineered microorganisms for effective production of environmentally friendly chemicals from renewable biomass. Proceedings of 3rd Annual Green Chemistry and Engineering Conference, Washington, D.C. Pp. 77-78.

Wang TT, Lee CF, Lee BH. 1999. The molecular biology of Schwanniomyces occidentalis Klocker. Critical Review in Biotechnol. 19(2): 113-143.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6 (13): 3901-3907.

Dohmen RJ, Strasser AWM, Martens FB, Dahlems UM, Hollenberg CP. 1990. Cloning of the *Schwanniomyces occidentalis* glucoamylase gene (GAM1) and its expression in *Saccharomyces cerevisiae*. Gene 95: 111-121.

Strasser A, Martens FB, Dohmen J, Hollenberg CP. 1992. Amylolytic enzymes producing microorganisms, constructed by recombinant DNA technology and their use for fermentation processes. US patent No. 5,100,794.

Claros MG, Abarca D, Fernandez-Lobato M, Jimenez A. 1993. Molecular structure of the SWA2 gene encoding an AMY1-related alph-amylase from *Schwanniomyces occidentalis*. Curr. Genet. 24: 75-83.

Piper P. 1996. Isolation of yeast DNA, Methods in Molecular Biology, 53:103-107.

SUMMARY OF THE INVENTION

The present invention provides the promoter clone discovery of a glucoamylase gene of a starch utilizing yeast strain *Schwanniomyces castellii*. The isolated glucoamylase promoter is an inducible promoter, which can regulate strong gene expression in starch culture medium.

An object of the present invention is to provide an isolated yeast promoter, which is native to Schwanniomyces castellii (ATCC 26077) and located upstream of and in control of a glucoamylase gene.

Another object of the invention is to provide an isolated yeast promoter that has a sequence of 1662 base pairs prior to the initiation codon of glucoamylase gene.

Yet another object of the invention is to provide a strong gene promoter that allows effective direction of transcription or expression of a gene of interest.

Another object of the invention is to provide a process of expressing a gene of interest in bacterial, yeast, mold, and plant/plant cell species.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration depicting an inverse PCR method for promoter clone isolation.

FIG. 2 is a photograph of a reverse gel image of PCR clones of *S. castellii* glucoamylase promoter.

FIG. 3 is a schematic illustrating the construction of a plasmid vector pGA2066.

FIG. 4 is the sequence of *S. castellii* glucoamylase promoter.

FIG. 5 is the sequence comparison of two *S. castellii* glucoamylase promoter sequences.

FIG. 6 is a schematic illustrating the construction of a plasmid vector pGA2100.

~~FIG. 7 is a schematic illustrating the construction of a plasmid vector pGA2101.~~

DETAILED DESCRIPTION OF THE INVENTION

The present invention comprises a promoter discovered in a starch utilizing yeast strain, *Schwanniomyces castellii* (ATCC 26077), which can completely hydrolyze and use starch in less than 10 hours. The novel promoter is related to an amylolytic enzyme, glucoamylase. The isolated glucoamylase promoter (GAM) clone has a length of 2184 base pairs, within which 1662 base pairs are putatively determined as the promoter region based on the open reading frame analysis. Within the 600-bp upstream of the glucoamylase initiation codon, there are putatively seven CAT boxes and ten TATA boxes, which play an important role in the regulatory mechanism of the GAM promoter.

The present invention illustrates that when fused to a reporter gene, glucuronidase gene (*gus*), the GAM promoter can regulate glucuronidase (GUS) expression in transformed *Saccharomyces sp.* The 1.0 kb GAM promoter shows stronger expression than the 1.5 kb GAM promoter in both glucose and starch culture medium. In addition, the *S. castellii* GAM promoter is an inducible promoter, which can regulate high gene expression at the presence of a starch while it can regulate minimal gene expression at the presence of glucose. The expression yield can be increased over twenty times when induced in a starch culture medium as compared to the glucose culture medium.

sub a2 However, other reports showed that a glucoamylase structure gene of a similar *S. castellii* strain (ATCC 26076), which is under the control of a native GAM promoter, was unable to express in the transformed *Saccharomyces cerevisiae* host while this glucoamylase gene was able to express under the control of *S. cerevisiae* promoters such as galactokinase (GAL1) promoter. This is probably due to the difference in the 5' flanking region of the glucoamylase gene of these two *S. castellii* strains. When compared to the GAM gene 5' end region of a *S. castellii* strain (ATCC 26076), it was found that there are sequence differences at positions 160-162 bps, 168-169 bps, and 288 bp using GAM76 as basis as shown in Figure 4(C), where GAM76 stands for the GAM

5' end flanking region of *S. castellii* (ATCC 26076) and GAM77 for the GAM 5' end flanking region of *S. castellii* (ATCC 26077). In GAM77, there is a sequence for CCATTATGGAT as compared to the difference of CATATGGTA in GAM76, which might cause inactivation of GAM76 in *S. cerevisiae*. However, there was no comparison made beyond 325 bps upstream of the initiation codon between two glucoamylase genes since the 5' end sequence beyond 325 bps upstream is not available for *S. castellii* (ATCC 26076).

The inducibility of the present GAM promoter provides an opportunity to regulate native or foreign gene expression in native or heterologous host strains with an inexpensive inducing agent, starch. Industrial fermentation/culture process can then use this cost-effective regulatory mechanism for certain type of metabolic pathway controlling and foreign protein accumulation.

For a clear and concise understanding of the specification and claims, including the scope given to such terms, the following definitions are provided:

PROMOTER: The expression of a gene is directed by a promoter, which is a DNA sequence and locates in the 5' region of a gene. A yeast promoter is a promoter sequence that will direct the transcription of a gene in yeast cells.

CONSTITUTIVE PROMOTER: The rate of gene transcription under such promoter is not regulated by an inducing agent, which can be a chemical compound, or a carbohydrate.

INDUCIBLE PROMOTER: The rate of gene transcription under such promoter is regulated by an inducing agent, which can be a chemical compound, or a carbohydrate.

PLASMID VECTOR: A DNA plasmid vector contains a replicon or an origin of replication able to autonomously replicate the plasmid DNA in the original host organism. A plasmid vector can also serve as both a cloning vector for DNA manipulation in a bacterial host and a shuttle plasmid vector for interested DNA expression in another host cell.

CLONING PLASMID VECTOR: Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which interested DNA sequences can be inserted for DNA manipulation purposes. Cloning vectors also contain a marker

gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide phleomycin resistance or ampicillin resistance.

EXAMPLE 1 PCR cloning of glucoamylase promoter of *S. castellii.*

To isolate the glucoamylase promoter, *S. castellii* cells were grown overnight in a culture medium containing yeast extract 1%, peptone 2%, and glucose 2%. Cells were then harvested and genomic DNA was isolated and purified from the culture using the spheroplasting method. Inverse PCR method was used to clone out the promoter region, as shown in Figure 1 where P1 is PCR reverse primer 1; P2 PCR forwarding primer 2; RE restriction enzyme site which can be cleaved both upstream of the glucoamylase (GAM) promoter and inside of the GAM gene; SCst *S. castellii.* PCR primers for the inverse PCR were designed based on the open reading frame of the GAM gene sequence of *S. castellii.* A 5' end over-hung sequence (italics) was designed to adapt restriction enzyme sites (underlined) such as Xba I and Sph I. The inverse PCR primers are listed as following:

Reverse primer GL1-C61:

5'-*GC <u>TCT AGA</u>* CAT ATG AGT AGT TTC CGT AGT AAT TGA-3'

Reverse primer GL2-C62:

5'- *GC <u>TCT AGA</u>* ATT ACT ATA CTT TTA ATC AGC TTC AGA-3'

Forwarding primer GL1-N64:

5'-*GAT <u>GCA TGC</u>* TAT CTT TAA TGA CTC TGC TGT CGA TGC -3'

Forwarding Primer GL3-N66:

5'-*GAT <u>GCA TGC</u>* TAG TTG TTA AAC CAC TGG TGG AAG GTG-3'

Inverse PCR method was used to isolate the promoter region. In this method, the genomic DNA was first digested with different restriction enzymes, such as Bcl I, BstB I, Hinc II, Hpa I, Sac I, and Xmn I, which locate within the 5' end of the glucoamylase gene region. Upon digestion, the DNA samples were purified and self-ligated using T4 DNA ligase, and the promoter region was subsequently cloned out by the inverse PCR reaction. Table 1 shows the reverse PCR reaction matrix, which pairs various sets of reverse primer and forwarding primer together.

Table 1. Inverse PCR primer pairing conditions for each digested and ligated genomic DNA samples.

PCR reaction No.	Restriction enzyme used before ligation	Inverse PCR primer pairing
1	Bcl I	GL1-C61; GL3-N66
2	Bcl I	GL1-C62; GL3-N66
3	BstB I	GL1-C61; GL3-N64
4	BstB I	GL1-C62; GL3-N66
5	Hinc II	GL1-C61; GL3-N66
6	Hinc II	GL1-C62; GL3-N66
7	Hpa I	GL1-C61; GL3-N66
8	Hpa I	GL1-C62; GL3-N66
9	Sac I	GL1-C61; GL3-N66
10	Xmn I	GL1-C61; GL3-N66
11	Xmn I	GL1-C62; GL3-N66

The inverse PCR reactions were conducted based on the primer pairing outlined in Table 1. After PCR reaction, the PCR products were separated in an agarose gel by electrophoresis. The inverse PCR results are shown in a reverse gel image in Figure 2, where lane number is correspondent to each inverse PCR reaction in Table 1 and lane S is the Hλ DNA size marker. The isolated GAM promoter clones are shown as dark bands

in the gel picture. Lanes 1, 2, 5, 6, 7, and 8 show strong bands, which correspondent to the ligated DNA samples previously cleaved by Bcl I, Hinc II, and Hpa I, respectively. The sizes of the PCR clones range from about 0.4 kb to 4.4 kb, and the strong bands range from about 1.7 kb to 2.3 kb.

EXAMPLE 2 Nucleotide sequence of GAM promoter sequence.

Sub Q4 PCR product No. 2 contains sufficient length (about 2.3 kb) of the GAM gene upstream sequence and the initial codon ATG of the GAM gene. The GAM promoter clone No. 2 was cloned into a unique cloning site containing 3' terminal thymidine (T) to both ends of a cloning plasmid vector pGEM-T (Promega, Madison, WI) to form pGA2066, as shown in Figure 3, where Amp is the ampicillin resistance gene; ColE1 is the origin for plasmid replication during gene manipulation in *E. coli* strains; f1 ori is the phage origin. Individual colonies were picked to confirm DNA insertion. Two of the randomly picked individual clones, pGA2066-21 and pGA2066-29, were sequenced to compare the identity of these two clones. The results showed that these two clones have the same sequences. In addition, other clones from PCR products No. 8 and 11 were also sequenced and they had the identical sequences as that of No. 2, and the sequence downstream of the initiation codon ATG is identical to the GAM1 gene. Therefore, the upstream of clone No. 2 has the GAM promoter region. Clone No. 2 in pGA2066-21 was chosen and completely sequenced from both ends, and has a length of 2182 bp, as shown in Figure 4(A) and Figure 4(B). The putative TATA box and CAT box are bold and underlined. There are seven CAT boxes and ten TATA boxes within 600 base pairs upstream of the initial codon. In addition, the open reading frame analysis indicates that the GAM promoter sequence locates between 485 bp to 2148 bp, between which there are 1662 bps for the GAM promoter and there is no long open reading frame.

EXAMPLE 3 Vector construction for GAM promoter analysis.

Sub Q5 To test the activity of the glucoamylase (GAM) promoter, a bacterial glucuronidase gene was fused to the 1.5 kb and 1.0 kb glucoamylase promoters. An episomal yeast plasmid vector pGA2028D was used, as shown in Figure 5. The GAM

promoter was cloned out from pGA2066-21, forming the 1.5 kb and 1.0 kb GAM promoters (GAM15 and GAM10). A 5' end over-hung sequence (italics) was designed to adapt restriction enzyme sites (underlined) such as Spe I at the 5' end and Hind III at the 3' end, using the following primers, respectively.

Forwarding primer GM15-N for 1.5 kb GAM promoter:

5'-*TCT AGA* *ACT AGT* GAT TTC TGA TTG ATT TGA GTT-3'

Forwarding primer GM10-N for 1.0 kb GAM promoter:

5'-*TCT AGA* *ACT AGT* TCT ATC AAA CTA CTC CAA ATA-3'

Reverse primer GM-C for both GAM promoters:

5'-*GGT ACC* *AAG CTT* CTT GCT TGT TAT AAT ACA GTC-3'

Figure 5 shows the constructed vector pGA2100 containing the 1.5 kb GAM promoter, and Figure 6 shows the vector pGA2101 containing the 1.0 kb GAM promoter, where 2 micron is a DNA replicon for plasmid replication in *Saccharomyces* strains; ColE1 is the origin for plasmid replication during gene manipulation in *E. coli* strains; f1 ori is the phage origin; *gus* is the bacterial glucuronidase gene; Tcyc1 is the transcription terminator; and Zeocin is the Zeocin resistance gene (Invitrogen, Inc, Carlsbad, CA). pGAM15 and pGAM10 stand for the GAM promoter with lengths of 1.5 kb and 1.0 kb, respectively.

EXAMPLE 4 Glucuronidase (GUS) expression regulated by GAM promoter in glucose culture medium.

A starch-degrading *Saccharomyces* hybrid yeast strain, obtained from James R. Mattoon of University of Colorado, was used as the host for plasmid transformation and promoter activity testing. A transformation kit (Invitrogen, Inc., Carlsbad, CA) was used for preparing competent yeast cells, which were subsequently used for the transformation of pGA2100 and pGA2101. After transformation, cells were plated onto YPD agar

medium plate containing glucose 2%, yeast extract 1%, peptone 2%, and antibiotic Zeocin (Invitrogen, Inc., Carlsbad, CA). After four-day incubation at 30°C, transformed yeast colonies were obtained on the selective culture plate and used for the GUS activity analysis.

Colonies were picked and intracellular protein samples were extracted using the glass-bead disintegrating method. Briefly, in this method single transformed colony was suspended in 300 µl of extraction buffer containing 50 mM sodium phosphate at a pH 7.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM beta-mercaptoethanol, and 0.1% triton X-100. The cells were then disrupted by acid-washed glass beads (average diameter 100 µm) on a vortexer. After cell disruption, the sample was centrifuged at 20,000×g for 5 minutes. The supernatant was saved for both protein and GUS activity assays. Protein was determined using a BioRad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) and glucuronidase activity using an enzymatic reaction in which a substrate 4-methylumbelliferul-beta-D-glucuronide (MUG) can be hydrolyzed by glucuronidase to a fluorescent compound 4-methylumbelliferone. One unit of glucuronidase activity is defined as the amount of glucuronidase that produces one pmole of 4-methyl umbelliferyl (MU) from MUG per minute at 37 °C. The specific activity of glucuronidase is calculated as the units of glucuronidase per milligram of total protein.

After transformation, positive yeast colonies were selected and grown in YPD plates containing glucose as the main carbon source and an antibiotic Zeocin. Transformed colonies were harvested and protein samples were prepared. Table 2 shows the results of glucuronidase specific activities of both clones transformed with pGA2100 and pGA2101, respectively. Two transformants are under the control of the GAM15 promoter, and four transformants are under the control of the GAM10 promoter. It is evident that GUS activity is detected in all the tested clones, but not in the none-transformed control cell, indicating that the isolated glucoamylase promoter is active in the heterologous host, *Saccharomyces sp.* The GUS expression under GAM10 promoter is higher than the one under the control of GAM15 promoter.

Table 2. GUS activity in transformed *Saccharomyces sp.* colonies grown in medium containing glucose 2%, yeast extract 1%, and peptone 2%.

Clone No.	Promoter	GUS specific activity (unit/mg)	Average activity (Unit/mg)
C*	-	6	6
1	GAM15	54	60
2		66	
3	GAM10	100	97 ± 15
4		99	
5		111	
6		76	

*Host cell without transformation of GUS expression vector

EXAMPLE 5 Glucuronidase expression regulated by GAM promoter in starch culture medium.

The GUS expression under glucoamylase promoter exhibits lower activity as shown in Table 2 when the culture medium contains glucose as the primary carbon source. In this test, transformed colonies were first grown in the medium contain glucose. After washing, cells were transferred into culture medium containing potato starch 2%, yeast extract 1%, and peptone 2% for GUS the expression tests. Results are shown in Table 3. After a 15-hour growth period in the starch medium, intracellular protein samples were obtained using the glass-bead disintegrating method and GUS activity was determined. GUS activity was detected in all transformed cultures. The highest GUS activity was 1405 U/mg-protein under the control of the GAM15 promoter, and 2123 U/mg-protein under the control of GAM10 promoter. When induced by starch at its highest expression level, the transformed GUS activity is as about 23-fold as the

activity in the glucose medium for the GAM15 promoter, and as about 22-fold as the activity in the glucose medium for the GAM10 promoter. These results indicate that the glucoamylase promoter is highly induced by starch, which can be used as an inexpensive inducting agent for gene expression regulation.

Table 3. GUS activity in transformed *Saccharomyces sp.* colonies grown in medium containing potato starch 2%, yeast extract 1%, and peptone 2%.

Clone No.	Promoter	GUS specific activity (unit/mg)	Average activity (Unit/mg)
C*	-	0.0	0.0
1	GA15	1394	980 ± 350
2		1405	
3		582	
4		854	
5		685	
6		963	
7	GA10	1398	1521 ± 327
8		1645	
9		2123	
10		1250	
11		1432	
12		1277	

*Host cell without transformation of GUS expression vector

EXAMPLE 6 Glucuronidase expression regulated by glucoamylase promoter in plant cells.

To test the functionality of glucoamylase promoter in plant cell cultures, the constructed plasmid vectors pGA2100 and pGA2101 were used in a transient

assay using plant cell protoplasting method. A 3-day old Nicotiana tabacum cell suspension was used for the preparation of protoplasts. Briefly, protoplasts were isolated by treating the suspension cells with a pH 5.8 solution containing 10 mg/l cellulase, 500 μg/ml pectoplyase (Kanematsu-Gosho, Los angeles, CA) and 0.4 M D-mannitol at 28°C for 20 minutes with a gentle shaking at 100 rpm. The protoplasts were then extensively washed with 0.4 M mannitol to remove cellulase and pectolyase. Finally, 1×10^6 protoplasts were resuspended in 0.5 ml of pH 5.5 electroporation buffer containing 2.38 mg/ml HEPES, 8.76 mg ml NaCl, 735 μg/ml CaCl2 and 0.4 M D-mannitol.

After addition of 20 μg superecoil plasmid DNA of pGA2100 and pGA2101, respectively, and 10 μg salmon sperm DNA as a carrier DNA, the protoplasts were them electroporated at a 300 volt pulse with 210 μF capacitor. The treated protoplasts were subsequently transferred in 7 ml of protoplast culture medium in a Petri dish and cultured for 48 hours at 28°C. The culture medium is a modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 4.3 mg/ml MS salt supplemented with 3% sucrose, 0.18 mg/ml KH_2PO_4, 0.1 mg/ml inositol, 1 μg/ml thiamine hydrochloride, and 0.2 μg/ml 2.4-dichlorophenoxyacetic acid (2.4-D), and 0.4 M D-mannitol.

The culture protoplasts were collected by gentle centrifugation and suspended in 100 μl extraction buffer containing 50 mM Tris-HCl pH 8.3, 227 mM NaCl, 1 mg/ml bovine serum albumin, and 1 mg/ml sodium azide. Protein samples were extracted by sonicating the protoplasts three times for 8 seconds with 30-second intervals. The protein samples were harvested by centrifuging the sonicated mixture at 15,000 g for 5 minutes. The supernatant was saved and protein concentration was measured by the Bio-Rad Protein Assay method (Bio-Rad, Hercules, CA). The glucuronidase activities were assayed using the same method as described in example 4. The glucuronidase activity results are shown

in the following table. The results indicate that *S. castellii* glucoamylase promoter can regulate the expression of glucuronidase in plant cells.

Table 4. GUS activity in electroporated Nicotiana tabacum cells.

Test No.	Promoter	Culture Medium	GUS Specific Activity (unit/mg)
Control*	-	Sucrose	10.2
1	GA15	Sucrose	49.2
2			36.0
3	GA10	Sucrose	60.0
4			51.2

*Host cell without transformation of glucuronidase expression vector.